Enhancing vaccination efficacy by application of in vitro preselected dietary fibers and lactic acid bacteria

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Impact of oral *Salmonella* vaccination and dietary interventions on fecal microbiota development of neonatal piglets

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Abstract

Scope: Restriction of antibiotics in livestock feeding, prompt the research interest towards alternative strategies for disease prevention and growth enhancement. The aim of this study was to determine whether vaccination and *Salmonella* Typhimurium (STM) challenge, in the presence and absence of immune stimulating dietary supplementation, influence vaccination efficacy and microbiota colonization. Piglets received either prebiotic long chain inulin-type fructan (lcITF) or a synbiotic combination of lcITF and the probiotic strain *L. acidophilus* W37 (LaW37). Additionally, we investigated if possible impact on microbiota changes are associated with improved host health during stress periods such as weaning and STM challenge. **Methods and Results**: Fecal samples from pre-weaning, post-weaning and challenge periods were characterized for their bacterial composition through 16S rRNA gene sequencing. Results revealed that significant enhancement of antibody titer against STM was associated with microbial composition. Early life supplementation with the synbiotic seemed to modulate piglets' fecal microbiota as abundance of lactobacilli tended to be increased on day 10 after birth. This effect was also observed on day 30 but not at other time-points. On day 30 the animals treated with the prebiotic had increased abundance of *Catenibacterium* which was also not observed at other time-points. After STM-challenge, the microbiota composition of the placebo group that received vaccination (CTRL/V) and synbiotic (lcITF/LaW37/V) group was more similar compared to the non-vaccinated placebo (CTRL/NV) and the prebiotic (lcITF/V) groups and were characterized with higher abundance of *Provetallaceae* population and lower *Lactobacillaceae* in their feces. **Conclusion**: Collectively, our results describe comprehensively the dynamics of fecal microbiota establishment in neonatal piglets under the influence of dietary interventions, weaning, vaccination and STM challenge. Moreover, we show that health-associated bacteria were promoted at specific time-points, during dysbiosis period, for both dietary interventions.

Introduction

Application of antibiotics in livestock feeding has been practiced for decades to prevent pathogenic infection and promote animal growth [1]. Although effective in livestock, the extensive use of antibiotics has contributed to the global threat of antibiotic resistant pathogens [2]. The global health issues associated with antibiotic resistant species have led to tighter regulations on antibiotics administration. In 2006, the European Union totally banned the sub-therapeutic use of antibiotics in livestock feeding [3] (http://europa.eu/rapid/press-release_IP-05-1687_en.htm). This ban, however, has led to increase use of antimicrobial agents and a rise of resistant *Salmonella* in pigs subsequently occurred [4]. Pigs are mostly asymptomatic but presence of *Salmonella* in pigs is an important risk for meat contamination. Currently, salmonellosis causes diarrheal diseases in over 130 million humans yearly (http://www.who.int/mediacentre/factsheets/fs139/en/) and is responsible for 9.3% of 225 foodborne outbreaks in Europe [5]. Therefore, there is an urgent need for alternative strategies to prevent infection with *Salmonella* in livestock. Vaccination and improvement of feed strategies to support immunity of livestock are two examples of these strategies.

Vaccination of piglets against *Salmonella* occurs via the oral route but is not fully effective as it confers only 20 to 50% protection [5, 6]. To improve efficacy of *Salmonella* vaccination, it has been proposed to combine vaccination with immune active food components [7]. Amongst the most extensively studied immune active agents are dietary fibers and lactic acid bacteria. Dietary fibers stimulate a stable and functional intestinal microbial community, and inulin-type fructans (ITF) have been shown to support bifidobacterial growth and activity [8]. Direct introduction of live *Lactobacillus acidophilus*, has been associated with enhanced health status [9, 10], improved resistance to diseases [11, 12], reduced shedding of pathogens [13], reduction of disease symptoms [14, 15], and support of intestinal immunity [7, 16, 17]. All these interventions, including vaccination, have been applied orally which implies that the vaccine and/or feed ingredients interact with the gastrointestinal microbiota of the piglets, thus

affecting the immune and metabolic status of the animals in later life [18-21]. Although this is well recognized, the effect of *Salmonella* vaccination and dietary intervention on microbiota development has, to the best of our knowledge, never been studied.

The colonization process of the intestines starts at birth, and in porcine livestock, the intestinal microbiota reaches an adult-like composition by 3 to 4 weeks post weaning. The adult pig microbiota is typically composed of genera *Clostridium*, *Blautia*, *Lactobacillus*, *Prevotella*, *Ruminococcus*, *Roseburia*, the RC9 gut group and *Subdoligranulum* [22-24]. Members of the families *Enterobacteriaceae* and *Bacteroidiaceae* have been reported to be amongst the most abundant at birth; however, their proportion decreases with weaning, whereas the proportion of *Prevotellaceae* increases, becoming the most abundant family at the post-weaning stage [25]. During the vulnerable neonatal phase, piglets are protected by maternal antibodies while their intestinal immune system develops and matures [26]. Interactions between the gut microbiota and the developing gut are considered critical during this stage, as any perturbation can potentially lead to an impaired immune function later in life [18, 27]. Vaccination is commonly performed during the pre-weaning period, but it is, to the best of our knowledge, unknown whether it could affect the microbiota colonization process. In addition, our understanding of the dynamics of microbiota development at the time of weaning might also lead to more informed strategies to prevent colonization by potential pathobionts post weaning. For instance, *Salmonella* colonization of piglets often occurs during this period [28] and is associated with increased shedding, spreading throughout pig herds and is, ultimately, contaminating pork meat [5]. In this context, feed supplements such as prebiotic ITF and probiotic *L. acidophilus* could be instrumental in supporting maturation of piglets' microbiota development and boosting immunity in early life. This, in turn, might lead to a better resistance of the animals against enteropathogenic infections via both increasing their response to vaccination against *Salmonella*, and protecting them against weaning associated stress.

In the present study, we assessed potential effects of specific pre- and synbiotic dietary interventions as well as oral vaccination before weaning on the early life development of piglets' gut microbiota under the influence of dietary interventions and oral vaccination. Furthermore, we assessed the possible protective effects of these treatments during a *Salmonella* challenge later in life by measuring antibody titer in blood and diarrhea daily occurrence and severity.

Material and methods

Ethical statement

The experimental protocol was designed in compliance with the guidelines for animal research, and experiments were performed under the DEC committee approval DEC 2012.III.05.041.

Supplements, vaccine and challenge compounds

Long chain inulin type fructans (Frutafit® TEX! Sensus, Roosendaal, the Netherlands) (lcITF) isolated from chicory roots was provided by Sensus (Roosendaal, The Netherlands) and is a polydispersed mixture of linear fructans oligomers and polymers linked by β(2-1) bonds. The number of fructose molecules determines the degree of polymerization (DP). Specific characteristics of the inulin used in our study had a DP ranging from 10 to 60 and was characterized by high-performance anion exchange chromatography coupled with pulsed electrochemical detection (HPAEC-PED), which was performed on an ICS5000 system (Thermo Fisher Scientific, Waltham, MA, USA), equipped with a Dionex CarboPac PA-1 column (2 × 250 mm) in combination with a Carbopac PA-1 guard column (2 × 50 mm) (Supplementary Figure S1).

The probiotic lactic acid bacterium *Lactobacillus acidophilus* W37 (LaW37) was obtained from Winclove Probiotics, Amsterdam, The Netherlands.

Salmonella Typhimurium (STM) strain DT12 (B; O1, 4, 5, 12) was isolated from a pig mesenteric lymph node (MLN) [29]. Inocula were

prepared as previously described [30] and were used to challenge the piglets. In short, bacteria were grown from glycerol stocks in Brain-Heart Infusion medium at 37°C until stationary phase. Cell count was confirmed with plating on Columbia Blood Agar medium.

Salmoporc STM® is an oral live attenuated porcine vaccine licensed in Europe (IDT Biologica, Dessau-Roßlau, Germany).

Experimental design of the piglet trial

All animals used in this experiment were housed at Trouw Nutrition (Trouw Nutrition Research & Development, Sint Anthonis, The Netherlands). Twenty-nine Hypor*Maxter new born female piglets were selected from 20 sows and cross-fostered 24 h after birth to ensure homogenization of microbiota and limit confounding factors such as genetic background and maternal antibodies. Piglets were randomly allocated to one of the following four treatment groups: placebo control non-vaccinated (CTRL/NV), placebo control vaccinated (CTRL/V), lcITF/V and lcITF/LaW37/V. Our study focused on prebiotic interventions with and without additional probiotic treatment, and we therefore decided not to include a probiotic-only group. Litter size was standardized to 12-14 piglets, and each litter was assigned to only one of the treatment groups to prevent contamination between treatments. The researchers and farm technicians were blinded for treatment when carrying out animal handling and analysis.

Sows and suckling piglets were housed in farrowing pens with controlled temperature and humidity. At weaning, piglets were transferred to a health care unit for individual housing with *ad libitum* access to water and feed. The synthetic diet produced by Trouw Nutrition contained low fiber as adapted from Houdijk *et al.* 1998 [31] (Supplementary Tables S1 and S2).

A 2 x 2 factorial design was followed so that each treatment and vaccination group (Table 1) could be compared to another one with only one variable parameter. LcITF and LaW37 supplementations were administered daily by oral gavage, starting on day 2 after birth. Glucidex 2 (Roquette

Corporate, Lestrem, France) and starch carrier (Winclove Probiotics, Amsterdam, The Netherlands) served as placebo for the control groups. LcITF and lyophilized LaW37 were prepared freshly and mixed within 1 h prior gavage at a respective concentration of 0.114 g/kg BW and $5x10^9$ CFU/ piglet.

Table 1. Treatment groups organized in a 2x2 factorial design

Group	Supplementation	Vaccination	STM Challenge	Number of animals
CTRL/NV	Placebo	No	Yes	7
CTRL/V	Placebo	Yes	Yes	6
LcITF	0.114 g/d/kg BW	Yes	Yes	8
LcITF/ LaW37	0.114 g/d/kg BW + $5x10^9$ CFU/d	Yes	Yes	8

Weaning of the piglets occurred on day 24 after birth and oral vaccination with one dose of Salmoporc STM ® (10^9 CFU) on day 25. Piglets were given three weeks to recover from weaning stress and develop their microbiota and immune system. As of day 52 after birth, piglets received a daily *Salmonella* challenge by oral administration of 10^9 CFU of STM DT12 (GD Animal Health, Deventer, The Netherlands) for three constitutive days: 52, 53 and 54. The study ended on day 55 when the animals were euthanized by an intra-cardiac injection of barbiturate.

Blood was collected five times, with sterile S-Monovette lithium-heparinized tubes (Sarstedt AG & Co, Numbrecht, Germany), from the jugular vein on days 23, 25, 42, 52, 55 (Figure 1), prior to any other handling of the animals, for antibody titer.

Feces were collected via rectal stimulation shortly after birth and a set time in the morning on days 10, 17, 23, 30, 51 and 55 after birth. Fecal samples were stored in sterile tubes at -20°C until they were processed.

Diarrhea was scored daily in the morning as previously described [32]. It was scored per litter prior to weaning and per animal post weaning.

The scores ranged from 0 to 3, 0 being the ideal well shaped stool with soft but solid consistency and 3 being a severe diarrhea with light colored watery defecation. The intermediate score of 1 was used to report a soft sticky stool, whereas a score of 2 represented stools with a liquid consistency.

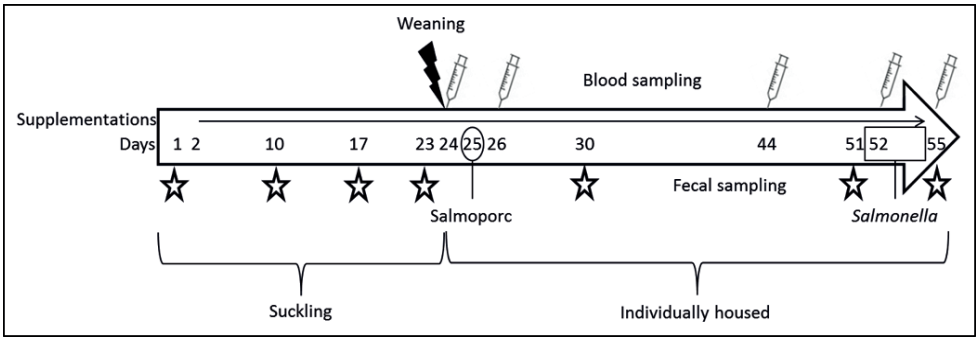

Figure 1. Experimental design. Female new born piglets were given daily oral gavages of placebo, lcITF or lcITF/LaW37 as of day 2 until sacrifice, on day 55 after birth. Weaned piglets (day 24) received Salmoporc oral vaccinated on day 25. Challenge with Salmonella Typhimurium (STM) DT12 was performed on days 52, 53 and 54, via oral gavage. Blood samples (syringes) were collected on the day of weaning, a day post vaccination, 20 days post vaccination, prior and post STM challenge. Fecal samples (stars) were collected shortly after birth, on days 10, 17, 23, 30, 51 and 55 after birth.

Serology

Blood plasma was collected after centrifugation at 2,000 × *g* for 10 min and was stored at – 80°C until further use. Anti-*Salmonella* antibodies were quantified using Salmotype Pigscreen ELISA according to manufacturer's instruction (Labordiagnostic Leipzig, Leipzig, Germany). The specific IgG levels were calculated using a reference standard method and are presented as S/P values. Stratification of the data was performed according to OD% with the following cut-off values: non-responders x < 40, low-responders 40 < x < 80 and high responders x > 100. Non-responders cut-off was indicated by manufacturer's instructions and based on non-vaccinated control group

responses. Cut-off value for low and high-responders was based on a naturally occurring gap in OD% of 20 between these two groups.

16S rRNA HiSeq sequencing of V4 region

Bacterial DNA was isolated from the fecal samples. Approximately, 0.1 g of fecal material was diluted in 600 μL of STAR buffer (Roche Diagnostics GmbH, Mannheim, Germany) and homogenized (Bertin Technologies, CNIM, Montigny-le-Bretonneux, France) (3 x 5.5 m/s for 30 s) with 0.1 mm diameter zirconia/silica beads (Sigma) and 3 glass beads (2.5 mm). Subsequently, homogenized samples were incubated at 95°C for 15 min and centrifuged for 5 min (4°C/13,000 g). Obtained supernatants were pooled and DNA was purified using the Maxwell R 16 Instrument (Promega, Leiden, The Netherlands) as described in detail by van Lingen et al (2017) [33]. Purified DNA was quantified using a DeNovix DS-11 (DeNovix Inc., Wilmington, USA) spectrophotometer, and aliquots of 20 ng/μL for each sample were prepared using nuclease free water for later polymerase chain reaction (PCR) amplification steps.

The PCR reactions were carried out in duplicates using 20 ng of DNA as template in each 50 μL reaction. One L from each of the primers 515-n and 806-n, targeting the V4 region of 16S ribosomal RNA (rRNA) gene region and uniquely barcoded per sample (10 μM each), was used along with, 1 x HF buffer (Finnzymes, Vantaa, Finland), 1 μL dNTP Mix (10mM each, Roche), 1 U Phusion® Hot Start II High Fidelity DNA Polymerase (Finnzymes, Vantaa, Finland) and 36.5 L of DNAse and RNAse free water. The amplification program included 30 s initial denaturation step at 98°C, followed by 25 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 10 s, elongation at 72°C for 10 s, and a final extension step at 72°C for 7 min. PCR products were visualized in 1% agarose gel, left to run in 135V for 25 min, and PCR product size (~290 bp) was compared to a 1 kb DNA ladder (ThermoScientific, Waltham, MA, USA). PCR products from each sample duplicate were pooled, purified using magnetic beads CleanPCR kit (CleanNA, Alphen aan den Rijn, The Netherlands) and eluted in 30 μL of nuclease-free

water (Qiagen). The DNA concentration of each sample was determined with Qubit BR dsDNA assay kit (Thermo Fisher Scientific, Waltham, MA, USA) and 100 ng of purified DNA were used for the construction of amplicon libraries. In total 210 samples were sequenced, distributed in 4 libraries of 70 samples, labeled with uniquely barcoded primers. Final amplicon pools were concentrated using magnetic beads and eluted in 25 μL of nuclease free water and the libraries were sent for Illumina HiSeq sequencing (GATC-Biotech, Konstanz, Germany).

Data normalization and statistical analysis

Data filtering and taxonomy assignment were performed using the NG-Tax pipeline [34]. Sequences were assigned to Operational Taxonomic Units (OTU) excluding low abundant OTUs (less than 0.1%) from each sample and taxonomy was assigned using the Silva_111_SSU [35]. In addition, two distinct in house assembled mock communities [34] were sequenced along with the study samples, to control for sequencing quality. The mock communities were compared with their theoretical composition for quality control as control for the different libraries. Analyses of alpha and beta- diversity were performed using the publicly available microbiome R package version 1.1.2 [36]. Microbiota data was analyzed using permutation multivariate analysis of variance (PERMANOVA), Adonis, Kruskal-Wallis, and diversity indices at each time point that were calculated using QIIME. False discovery rate (FDR) –referred to as *p*-value in the results section- with conditional effect, was used to determine the significance of explanatory variables. The differences in relative abundance of specific microbial taxa across time were assessed with the nonparametric Wilcoxon Rank sum test. Sample groups containing less than 20 samples were bootstrapped followed by 100 permutations. Results from diversity analysis across time were tested for their normality with Shapiro-Wilk's test, and whiskers plots were created in PRISM5. Principal response curve analysis was performed as implemented in CANOCO 5 [37], to test for treatment effects. Different analyses were performed because during preweaning animals were separated in three

groups (Placebo, LcI,LCsdf) and post weaning in four groups [38]. For non-parametric *t*-tests, reads were transformed to their relative abundances and tests were carried out with 999 permutations using QIIME. Diversity index group comparisons were done in GraphPad Prism 7.0a (GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance was determined using the Benjamini–Hochberg false discovery rate (FDR).

Antibody titers were not normally distributed as confirmed by D'Agostino & Pearson normality test and were further analyzed using Kruskal-Wallis followed by Dunn's multiple comparison test in GraphPad Prism version 7.0a (GraphPad Software). As results of Dunn's test, *p*-values of 0.05 or smaller were considered statistically significant and *p*-values between 0.05 and 0.1 were defined as a trend. Data are expressed as mean ± standard deviation (SD).

Diarrhea scores were tested in a Proc MIXED procedure of SAS 9.3 Software Version 13. (SAS Institute Inc., Stata Corporation, College Station, TX, USA) according to SAS/STAT 9.3 User's Guide using the following equation: $Y_{ijk} = \mu + T_i + S_j \times T_j + e_{ijk}$ where T is the treatment effect for each group (1,2,3,4), and recipient sows that foster the piglet is taken as a random factor. Fecal consistency was analyzed with a $\chi 2$ homogeneity test of the GENMOD procedure in SAS.

Results

The aim of this study was to determine whether vaccination and *Salmonella* Typhimurium (STM) challenge, in the presence and absence of immune stimulating dietary supplementation, influence vaccination efficacy and microbiota colonization. We followed the development of neonatal piglets from birth, for 55 days, to investigate whether dietary interventions were associated with alterations in the microbial communities in the gut during early life, weaning, vaccination, and pathogen challenge. Piglets received either prebiotic long chain inulin-type fructan (lcITF) or a synbiotic combination of lcITF and the probiotic strain *L. acidophilus* W37 (LaW37).

First, we confirmed that age was the main driving factor behind the development of the piglets' microbial communities prior to and post weaning as determined by both phylogenetic (Unifrac) based distances (Figure 3.A; and Supplementary Figure S2.A) and non-phylogenetic dissimilarity index (Bray-Curtis) (Supplementary Figure S2.B). Distinct differences between the microbiota composition of the samples prior and post-weaning indicate the pivotal role of the diet which was further confirmed by alpha diversity measured by phylogenetic index (Supplementary Figure S3 and S4), where post weaning timepoints reveal more diverse communities on response to the more complex food they receive (Supplementary Figure S3).

Enhanced vaccination efficacy and piglets' health with supplementations

The immune status of the piglets and vaccination efficacy were investigated as measurement of possible health associated effects of the dietary interventions. Levels of specific antibodies against STM in piglets' blood were used as measure for support of vaccination efficacy. Only the animals that received the combination lcITF/LaW37 had a significant increase of antibodies (Table 2). This was observed as of day 52 after birth (p=0.021) and was significantly enhanced upon STM exposure. It resulted in an antibody titer twice as high than in CTRL/V group, although not statistically significantly, and was significantly higher (p=0.0031) than in CTRL/NV.

Table 2. Antibody titer, presenting the percentage of animals which classified as responders and quantitative data prior and post STM challenge.

	Day 52			Day 55		
	% responders	Average antibodies	SEM	% responders	Average antibodies	SEM
CTRL/NV	0	0,12 [a]	0,04	0	0,18 [a]	0,05
CTRL/V	0	0,19 [a]	0,02	33	1,30 [a]	0,40
lcITF/V	13	0,30 [a]	0,06	25	0,37 [a]	0,06
lcITF/LaW37/V	25	0,62 [b]	0,09	75	2,74 [b]	0,38

To evaluate protection of the vaccinated piglets, we scored salmonellosis associated diarrhea occurrence and severity during challenge with STM on days 52, 53 and 54 after birth. Most piglets were asymptomatic during the challenge. Animals in the CTRL/V and lcITF/LaW37/V groups experienced low incidence of salmonellosis associated diarrhea (Figure 3). This was different in the CTRL/NV and lcITF/V groups in which respectively 23% and 15% of the piglets had salmonellosis associated diarrhea, however, this difference was not significant compared to CTRL/V and lcITF/LaW37/V groups.

Diarrhea is a symptom of weaning stress [39], and we used daily diarrhea scores to evaluate piglet's health from the day of weaning (day 24 after birth) until the end of the study (day 55). In the placebo groups, 40% of the piglets experienced diarrhea lasting three to 11 days within the first two weeks post weaning (Figure 3) This diarrhea episode could not be prevented by lcITF/LaW37 but it was shorter and lasted at most five days. Strikingly, piglets supplemented with lcITF alone did not experience post-weaning diarrhea, and diarrhea scores were significantly lower than the scores in the placebo groups at all times.

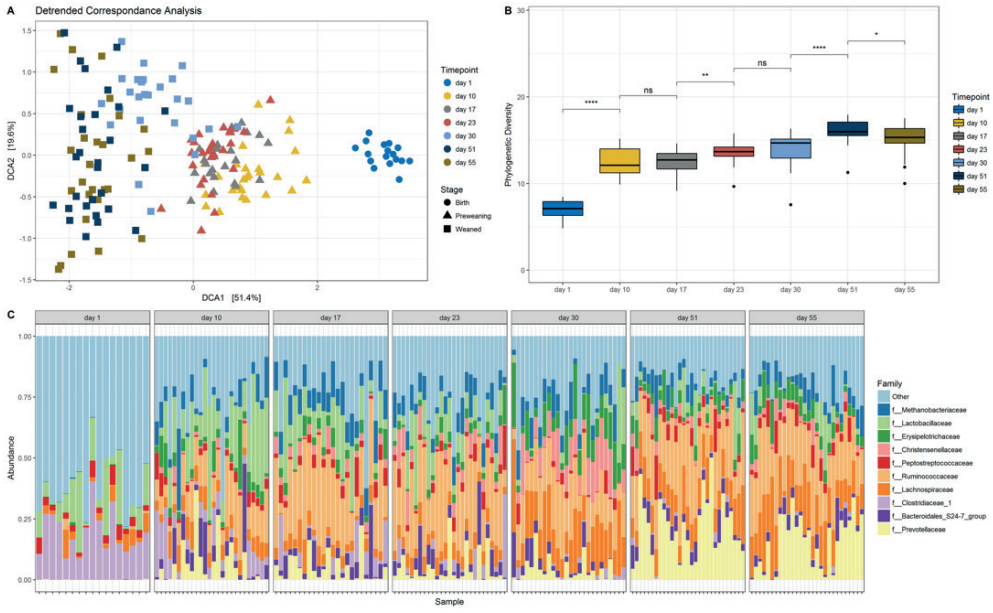

Figure 2. The development of microbial communities in neonatal piglets from birth to day 54 after birth. A) DCA plot illustrating the succession of microbial development in piglets. Percentages of variation are indicated for each axis B) Phylogenetic diversity of the developing microbial communities of piglets C) The microbial composition of neonatal piglets within each timepoint

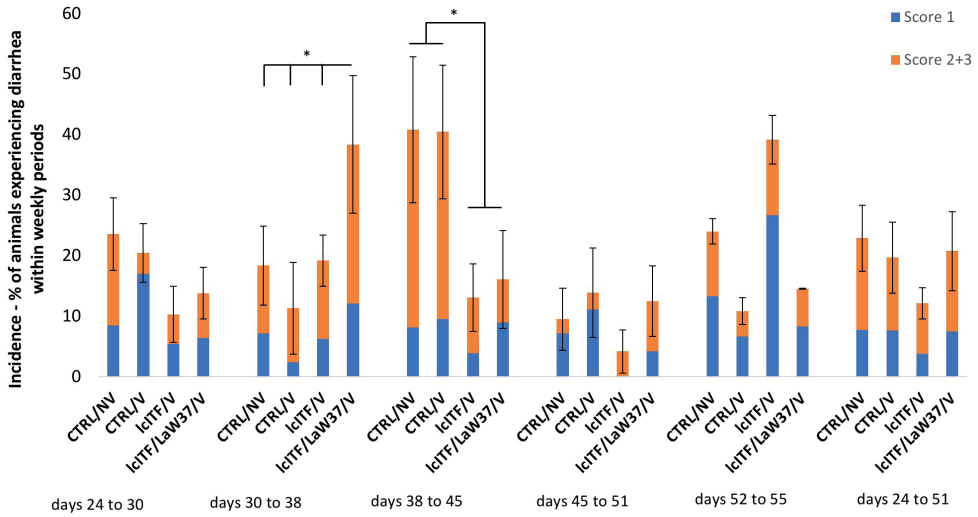

Figure 3. Diarrhea scores after weaning were impacted by the dietary interventions. Incidence of diarrhea is expressed as % of animals, within each treatment group, experiencing low (score 1) to severe (score 3) diarrhea. Incidence is shown per weekly period following weaning (day 24 after birth) and challenge perid with *Salmonella* Typhimurium is presented separately (days 52 to 55 after birth). Statistical significances are presented per week where * is $p<0.5$ as calculated with MIXED procedure of SAS. CTRL/NV = non-vaccinated placebo control group; CTRL/V = vaccinated placebo control group; lcITF = long-chain inulin; LaW37 = *Lactobacillus acidophilus* W37.

Effects of pre- and synbiotic dietary interventions on gut microbiota depended on the developmental stage of the piglets

We investigated whether dietary interventions would modulate changes in microbiota associated with weaning and the concomitant vaccination. Therefore, we evaluated the influence of the dietary interventions on early life microbiota development, prior to weaning, and how it changes as weaning occurs.

Prior to weaning, animals were distributed within three groups, a placebo group and two dietary intervention groups receiving either lcITF alone or lcITF combined with LaW37. A clear separation can be observed between the samples collected on day 10 (R^2=0.08, p=0.001) with those collected on day 17 and 23 (Supplementary Figure S5.A). In order to address more specifically the evolution of microbial composition under the influence of the different dietary interventions throughout time, principal responses curve analysis (PRC) was performed (Supplementary Figure S6 and Table 3). Although the model did not present a significant effect, it revealed that the genus *Lactobacillus* was more abundant in the lcITF/LaW37 group compared to the placebo and lcITF groups. The OTUs that were mainly affected belonged to the genus *Lactobacillus* (Supplementary Figure S7). Differences weren't significant for other two timepoints and phylogenetic diversity did not show any differences.

Table 3. Results from Principal Response Curve during preweaning and postweaning timepoints

PRC summary	Pre-weaning			Post-weaning		
Set	% Explained	F	P	% Explained	F	P
PRC.1	37.4	0.5	0.01	43.9	0.4	0.308
PRC.2	22.8	0.4	0.09	32.5	0.4	0.512
PRC.3	12.7	0.3	0.7	12	0.2	1
PRC.4	7.5	0.2	1	5.4	0.1	1

Weaning is considered to be the most sensitive period as piglets tend to develop enteric infections. In the present study piglets were weaned on day 23 and received an oral vaccine or a placebo on day 25. Samples were collected on day 30, one week after weaning, to assess possible differences in microbiota composition, driven either by vaccination or the combination of vaccination and dietary intervention. Introduction of solid food had a strong overall effect, regardless of the treatment group. As observed on Supplementary Figure S8.A, with weighted Unifrac distances, samples from day 30 separate from samples collected on post-weaning timepoints but also with day 51 and 55. and that specific enrichment in members of the *Christensenellaceae* family on day 30 was responsible for this separation (Supplementary Figure to add).

Next, we evaluated the effect for the different treatment groups during the post weaning timepoints with the PRC. Contrary with the preweaning timepoint, model revealed a significant effect for the differently treated groups and major differences can be observed on days 30 and 55, but not on day 51. On day 30 taxa which are differentially abundant are Lactobacilli from the lcITF/LaW37/V.

In accordance, Adonis analysis of variance presented significant differences on day 30 after birth, as different treatments significantly impacted fecal microbiota (R^2=0.20, p=0.007) (Table 4). It is apparent that dietary interventions were the main influencers for the differences observed on day 30 without influence of the vaccination. The lcITF/LaW37/V group was characterized by higher abundances of bacteria from the genus *Lactobacillus*, while the lcITF/V group presented higher abundances of *Catenibacterium* (Figure 4.B and 4.C). This significant effect of dietary interventions observed on day 30 was transient as no separation could be further observed on day 51.

Taken together, these data revealed that vaccination did not impact microbiota development but that dietary treatments did, although these effects could only be observed at specific time-points.

Table 4. Effects of different treatments during pre-weaning (dietary intervention) and post-weaning (dietary intervention and vaccination) with PERMANOVA and Adonis multivariate analyses of variance within each time-point. Asterisks indicate significance for the effects.

	Pre-weaning			Post-weaning		
	Day 10	Day 17	Day 23	Day 30	Day 51	Day 55
Treatment	R^2=0.08 p=0.4	R^2=0.09 p=0.26	R^2=0.04 p=0.68	R^2=0.2 p=0.015	R^2=0.09 p=0.62	R^2=0.41 p=0.001*

Bacterial communities are differently affected by the STM infection depending on the treatments

On the final stage of this trial, animals from all the groups were orally infected with STM. Challenge was applied daily for three consecutive days after which fecal samples were collected. Interestingly, a strong effect of the different treatments could be observed on day 55 as suggested by the PRC (Supplementary Figure S9 and Table 3). The groups lcITF/LaW37/V and CTRL/V shared a more similar microbiota composition, which significantly differed from the CTRL/NV and lcITF/V groups (Figure 5). This difference between the groups was observed at the level of *Prevotellaceae* family. LcITF/LaW37/V and CTRL/V groups presented high mean abundances of members from the *Prevotellaceae,* 0.23% and 0.26% respectively, while the CTRL/NV and lcITF/V groups had low mean abundances of 0.04% and 0.1% (Figure 5D). The opposite was observed for the *Lactobacillaceae* family (Figure 5D), low in the lcITF/LaW37/V and CTRL/V groups and high in the CTRL/NV and lcITF/V groups, although not significantly. This separation between the groups was further confirmed using Adonis analysis. The lcITF/LaW37/V and CTRL/V groups shared high similarities (Figure 5.A and 5.D), and the CTRL/NV and lcITF/V groups had a significantly different composition than lcITF/LaW37/V and CTRL/V (R^2=0.41; p=0.001) (Table 3). The OTU's which were responsible for the separation belonged to the taxa from Prevotella1, Phascolarctobacterium, Prevotellaceae_NK3B31_group and Lactobacillus

(Figure 5.B). This was further confirmed at the genus level (Figure 5.C).

Microbiota composition was most significantly impacted by the STM challenge. The changes observed for the lcITF/LaW37/V synbiotic group were more similar with the CTRL/V group in comparison with the lcITF/V and CTRL/V groups. Taken together, this suggests that lcITF might have suppressive effects on the vaccination as upon STM challenge the dysbiosis state of the piglets was more pronounced in non-vaccinated animals and vaccinated animals that received lcITF than in vaccinated animals and vaccinated animals that received lcITF/LaW37.

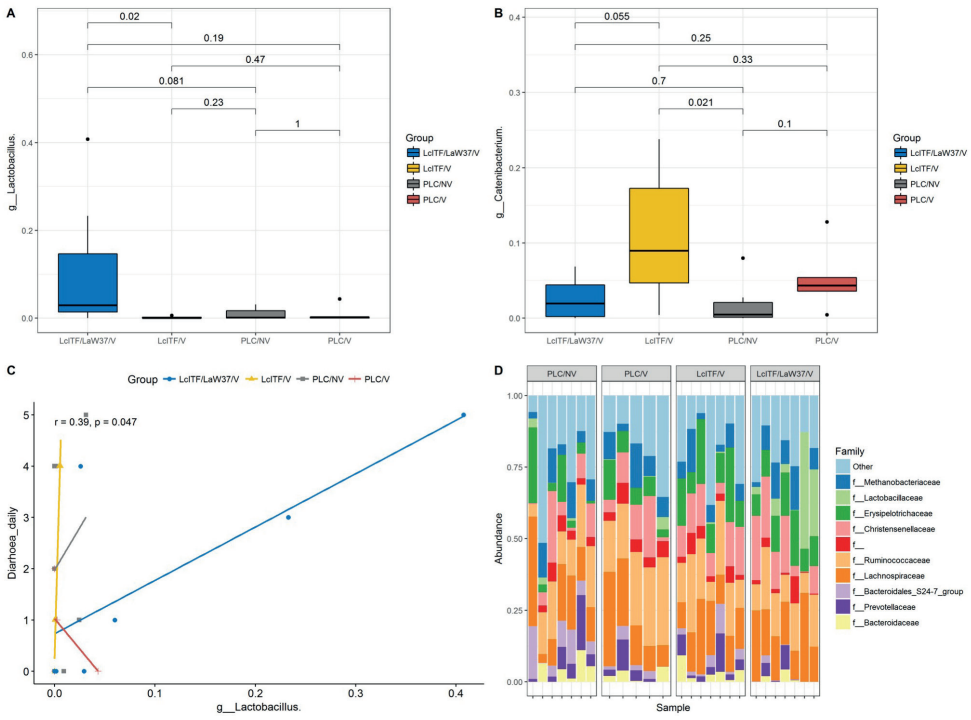

Figure 4. Dietary intervention but not vaccination affect the microbial development of piglets one week after weaning, on day 30. Relative abundances of A) the genus *Lactobacilus* and B) *Catenibacterium* on day 30 one week post weaning/vaccination C) Composition of the microbial communities between the four treaded groups on day 30. The top-10 families are annotated on the right legend.

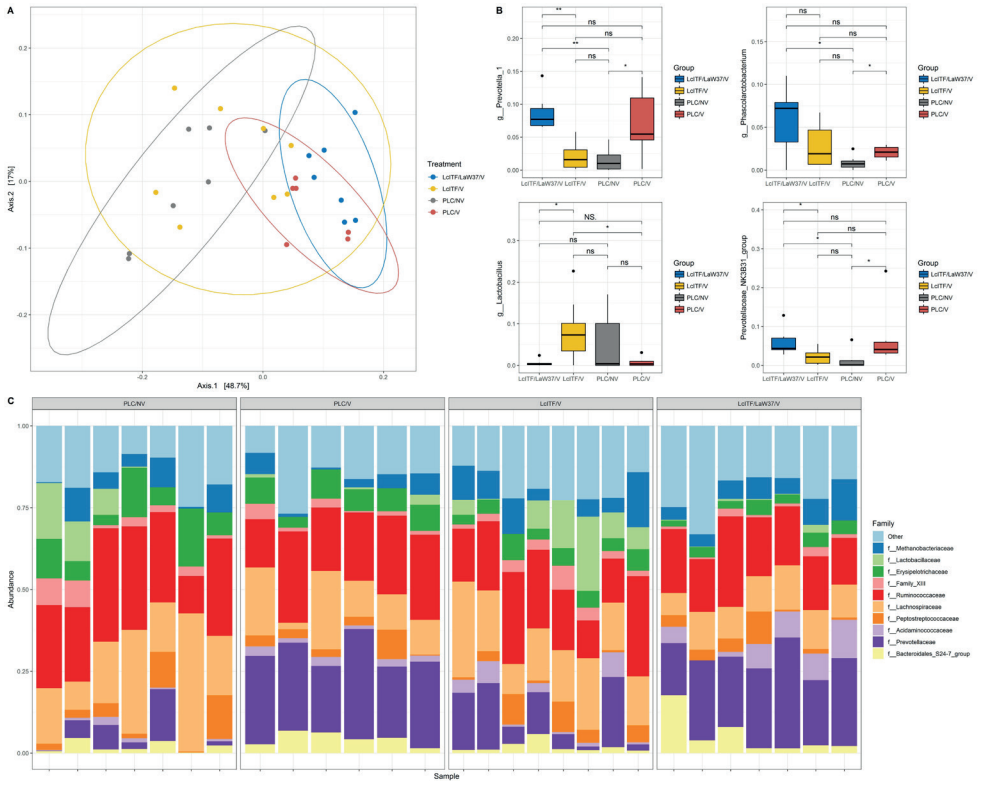

Figure 5. The effect of STM infection on the microbial composition of the piglets after three days challenge with the pathogen, on day 54.
A) PCoA plot using the weighted Unifrac distances between the four treated groups. Separation can be explained by the different treatment groups (Adonis R^2=0.41; *p*=0.001) B) The four taxa which drive the variation between the treatment groups C) Composition of the microbial communities between the four treaded groups on day 30. The top-10 families are annotated on the right legend.

Microbiota composition is associated with lower STM-induced diarrhea and higher antibody titer

To confirm lcITF might have suppressive effects on the vaccination we further investigated whether the differences between microbiota compositions of the different groups were correlated with the piglets' immune response. We observed lower diarrhea severity, as indicated by lower scores, as well as lower occurrence during challenge in CTRL/V and lcITF/LaW37/V groups (Figure 6). Moreover, these two groups were the only one to respond to the vaccination as their antibody titer increased upon secondary exposure (*i.e.* STM challenge). The opposite was observed for the CTRL/NV and lcITF/V groups. Interestingly, a negative correlation was found between antibody titer and diarrhea as shown Figure 6 (R^2=-0.3; p=0.06). The taxa which was highly correlated with the occurrence of diarrhea was *Dorea sp*. but no significant difference was detected between the treatment groups. Although the correlation found is weak it is in line with the overall clustering consistently observed that separates CTRL/V with lcITF/LaW37/V from CTRL/NV and lcITF/V confirming that lcITF might have lowering effects on STM oral vaccination.

Finally, we investigated whether microbiota composition of the animals with the highest antibody titers was outstanding and if it could explain for higher vaccination efficacy. As previously described, 75% of the animals that received lcITF/LaW37/V produced high amounts antibodies against STM while 33% and 25% of the animals in the CTRL/V and lcITF/V groups responded upon secondary exposure to STM. Therefore, we stratified the animals based on their antibody titer on day 55 as being high-, low- and non-responders towards the vaccination. As shown on Figure 7, a positive correlation between microbiota composition and the status of responders was observed (R^2=1.15; p=0.035).

A striking finding was that the microbiota shift observed on day 30 in animals treated with lcITF/LaW37/V was correlated with lower STM-induced diarrhea (p=0.026) (Figure 7). This suggests that the transient effect of lcITF/LaW37/V on microbiota composition during weaning stress could

have beneficial health effects later in life.

Because antibody titer was already enhanced prior to STM challenge, on day 52, in the lcITF/LaW37/V group, we further looked into possible microbiota changes prior to STM-challenge that could serve as microbial biomarkers and provide leads to explain enhancement of vaccination efficacy by lcITF/LaW37. However, no correlations could be found between microbiota composition and antibody titer suggesting that strong immune responses observed in the synbiotic group might also derive from other mechanisms than only microbiota changes.

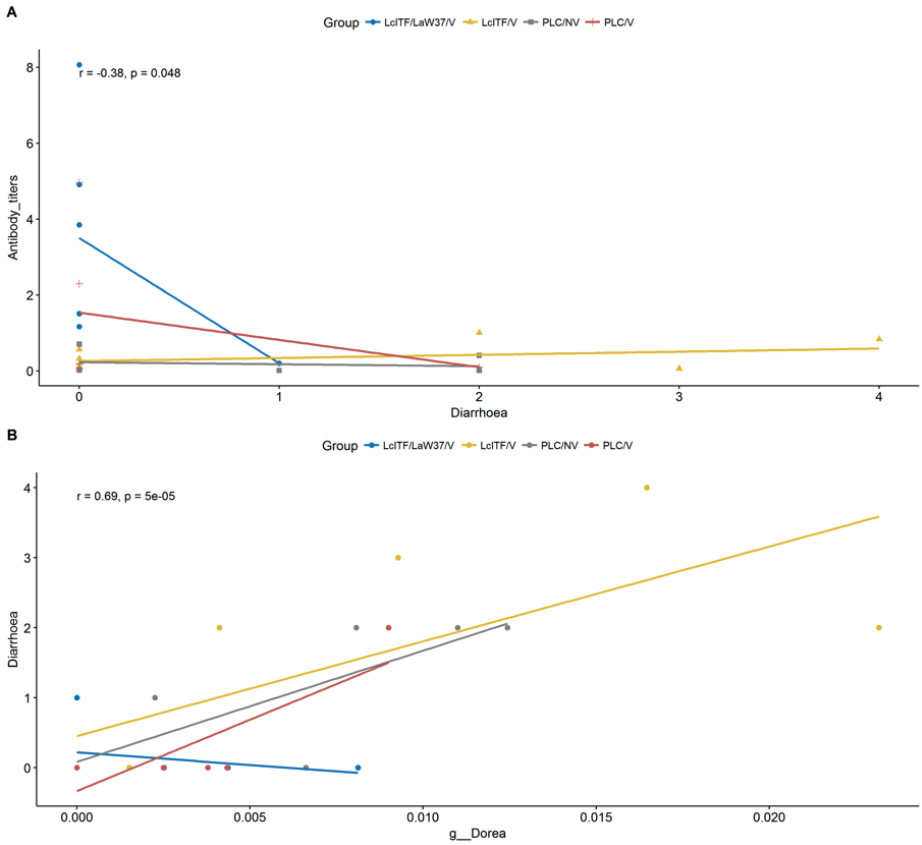

Figure 6. Correlation on day 55 between A) daily diarhea scores and antibody titters and B) between Dorea genera and daily diarrhea scores

Discussion

Effects of the residential gut community on the development of the immune system has been well described [40], however the interplay between gut microbiota and vaccination has not yet been fully explored, especially in livestock animals. This is of importance, as any interference with microbiota composition might influence immunity and metabolism in later life [18]. Within this context, the present study examined the effects of dietary interventions on piglets' microbiota throughout weaning, vaccination against *Salmonella* Typhimurium (STM) and STM challenge. The development of gut microbiota was monitored for a period of 2 months based on fecal community profiles obtained from NGS of PCR-amplified 16S rRNA gene fragments. Piglets were weaned on day 24, vaccinated on day 26 and challenged on days 52, 53 and 54 after birth to identify possible taxa associated with vaccine-mediated protection. Results from fecal samples suggested that weaning and STM-stress, but not vaccination itself, affected fecal microbiota composition. The subtle effects observed with the pre- and synbiotic dietary interventions on gut microbiota were specific and depended on the developmental stage of the piglets.

Vaccination was given orally to newly weaned piglets and did not lead to any change in microbiota composition as compared to non-vaccinated animals. In line with a large body of literature, we observed a significant shift between pre- and post-weaning fecal microbiota composition. Weaning is indeed known to induce important intestinal dysbiosis [39]. However, by the age of 51-days old, the microbial Alpha diversity of all piglets resembled adult, sow-like diversity. This matches developmental studies reporting on piglets core microbiota [23]. Interestingly, we could pin point that members of the *Christensenellaceae* family were responsible for the separation of samples on day 30. Some of these bacteria are known butyrate producers are associated with pigs' health Taken together these data show that gut microbiota development of the piglets throughout the whole study was not impaired by vaccination nor by any of the dietary supplementations.

Effects of dietary intervention were observed during dysbiosis periods. The most significant impact on microbiota composition later in life when animals received the STM-infection. Challenge with STM induced loss of diversity in all the groups, with Alpha diversity being lower for all groups on day 55 compared to day 51. Because this diversity dropped so abruptly it is safe to assume that the effect was due to challenge rather than normal development feature. This indeed matches previous findings [43, 44]. Moreover, differences in Alpha diversity were also observed between the groups and was lower in CTRL/NV and lcITF/V groups than in CTRL/V and lcITF/LaW37/V groups. *Prevotellaceae* and *Lactobacillaceae* were driving these differences and bacterial OUTs Prevotella1, Phascolarctobacterium, Prevotellaceae_NK3B31_group and Lactobacillus contributed to the variations. Changes in *Prevotellaceae* were previously observed in pigs infected with *Salmonella* [44]. The other time-point at which significant differences were observed between the groups was when piglets experienced weaning-induced stress. Piglets fed the synbiotic lcITF/LaW37/V had higher abundance of *Lactobacillus* than the other treatment groups. This is in line with a previous intervention, with lactobacilli, done at weaning, which induced ileal and colonic changes in pigs' microbiota [45]. Also observed after weaning, the piglets fed the prebiotic lcITF had higher abundance of *Catenibacterium*, a fiber degrading bacterium previously reported to be increased in pig's microbiota fed resistant starch [46]. Interestingly, piglets fed lcITF, after weaning, experienced less diarrhea suggesting that *Catenibacterium* might be health-associated. Interestingly, members close to this genus were reported to convert isoflavonoids into equol, an health-associated SCFA [47].

Increased abundances of *Lacobacillaceae* members in feces is associated with diarrhea and different dietary interventions are responsible for this depending on the time-point. The first time-point at which lactobacilli were increased in feces was on day 10 for the piglets fed lcITF/LaW37. Although this effect was very subtle, as it could not be picked up by all analysis performed, it shows that only the animals specifically fed

LaW37 as supplement had enhanced abundance of lactobacilli. It is likely that the very immature microbiota of these neonate piglets could only handle the settlement of a lower number of lactobacilli in the ileal, the rest being excreted in feces. This transient effect attenuates as the microbiota matures and no differences were observed at other time-points pre-weaning. Weaning stress, as measured on day 30, revealed higher abundances of *Lactobacillus* in feces of animals fed lcITF/LaW37. No differences were observed further on, but after STM-challenge on day 55, animals in the CTRL/V and lcITF/LaW37/V groups had lower relative abundance of *Lactobacillaceae* and of the OTU *Lactobacillus*. Interestingly, these data on lactobacilli are in line with scoring of diarrhea. On day 10, piglets were suckling and kept in herd, therefore diarrhea was not measured at this period for individual animals, but for the heard and no statistical significance could be found. However, on day 30 diarrhea was higher for piglets fed lcITF/LaW37 and on day 55 it was the opposite, moreover correlations analysis revealed that lactobacilli were positively correlated with diarrhea on day 55. An explanation is that fecal samples represent more a colon-like composition, where lactobacilli are low abundant in healthy situation [48]. However during dysbiosis period, like weaning-induced stress or STM challenge, feces composition will represent more the composition of the small intestine, where lactobacilli are typically more abundant [23]. *Salmonella* is known to invade ileal mucosa provoking diarrhea [49] therefore inducing loss of microbial diversity. Ileal microbiota composition can then be measured in fecal samples within the first days of an STM infection [43, 44].

Dietary lcITF intervention specifically reduced post-weaning diarrhea but not STM-induced diarrhea. Incidence of diarrhea is an important consequence of intestinal dysbiosis that occurs during both weaning and STM-induced stress. In this study, we found that supplementation with lcITF significantly improved piglet's health on that aspect during weaning-stress compared to other treatment groups. Interestingly, this was not the case during STM-induced diarrhea. Because animals were all tested STM-free at the start of the challenge, weaning-associated diarrhea episodes

were not due to salmonellosis. Most of the studies conducted during the weaning transition have reported a decrease in abundance of *Lactobacillus* spp. in favor of *Clostridium* spp., *Prevotella* spp. or facultative anaerobes such as *Proteobacteriaceae* [39]. Although *Catenibacterium* was specifically increased and might be contributing to protect piglets against weaning/associated diarrhea, sequencing of 16S rRNA gene fragments amplified from piglets' feces might lack sensitivity and specificity to unravel more responsible agents for the beneficial effects observed with lcITF. Our data suggest that supplementation with lcITF was most efficacious to prevent weaning-stress induced diarrhea which could be linked to higher abundances of fiber degrading bacteria but not STM-induced diarrhea.

Animals that received CTRL/V and lcITF/LaW37/V were more protected against salmonellosis but microbiota was not solely responsible for this. These two groups clustered together with increased antibody titer against STM, higher Alpha diversity and lower diarrhea frequency with shorter duration as compared to the CTRL/NV and lcITF/V groups. Feces of piglets treated with CTRL/V and lcITF/LaW37/V was typically characterized by higher relative abundance of *Prevotellaceae* and lower relative abundance of *Lactobacillaceae* than the CTRL/NV and lcITF/V groups. CTRL/V and lcITF/LaW37/V had higher abundances of the OTUs *Phascolarctobacterium*, *Akkermansia Prevotella1* and *Prevotellaceae_NK3B31_group*. This is in line with previous studies were the health associated butyrate producer *Phascolarctobacterium* was found in high abundance in feces of pigs fed resistant starch and other dietary fibers [46, 50]. Moreover, *Prevotella* typically colonizes the cecum and colon of healthy pigs [48] and higher levels of *Prevotellaceae* have been associated with health [51]. Also, lower abundance of the members of the *Prevotellaceae* is a characteristic consequence of STM infection in pigs. This corroborates the observations made in our study where groups with higher relative abundance of *Prevotellaceae* also experienced a stronger systemic reaction towards the STM-challenge as measured by increased vaccination efficacy which is in accordance with the diarrhea results of the present study. Salmonellosis diarrhea was higher

in lcITF/V with CTRL/NV groups that did not develop systemic immunity against STM after vaccination as measured by absence of circulating specific antibodies post challenge. In fact, we found a correlation between microbiota composition, diarrhea and antibody titer on day 55. Previous studies about lactobacilli effects on oral vaccination efficacy have shown promising results in line with ours [15, 52, 53] although these effects seem to be strain and vaccine specific [54]. Separation of the animals according to their response to the vaccine (high- low- non-responders) also indicated that levels of antibody titer and microbiota are linked. However, these correlations were weak and as no other link could be found between vaccination efficacy and microbiota at other time-points, it seems that changes in fecal microbiota cannot solely explain the doubled antibody titer measured in lcITF/LaW37/V as compared to the CTRL/V group which was similarly protected against STM-induced diarrhea and dysbiosis.

The suppressive effect of lcITF supplementation on vaccination efficacy was not expected. Earlier findings were that lcITF supports Hepatitis B vaccination by increasing Th1 cells [55], and STM vaccination is also known to be Th1 driven. However, short-chain ITF was found, in the study of Vogt *et al.* 2017, to have suppressive effects against Hepatitis B vaccination. It should be noted, however, that there are major differences between this study that targeted systemic vaccination of young adults and our design with oral vaccination of very young piglets. Taken together, these discrepancies might suggest that lcITF exerts Th1-driven effects only in the presence of an matured immune system and/or not at mucosal level, or that different degradation patterns occur in piglets receiving lcITF dietary intervention leading to the degradation of lcITF into molecules that have a suppressive effect on vaccination efficacy, as previously observed for other types of ITF [55]. However, dietary lcITF intervention was not associated, until challenge, with impaired general health.

In conclusion, neither neonate dietary interventions nor vaccination during weaning stress, provided in an appropriate dosage, impaired the piglets' immune and microbiota development. Interestingly, prebiotic

and synbiotic interventions were able to substantially modify later life immunity by respectively suppressing or supporting vaccination efficacy. Moreover, periods of stress such as weaning and STM-challenge were best to unravel subtle effects of neonate dietary interventions. Intervention with lcITF protected against non-STM driven diarrhea, which might indicate a protective effect in other regions of the intestine than in the ileum. Addition of LaW37 to lcITF drastically changed the effect of lcITF on vaccination efficacy, which was strongly enhanced. LaW37 is likely to drive changes in vaccination efficacy but the mechanisms behind this effect do not solely rely on changes in fecal microbiota composition.

Supplementary information

Figures

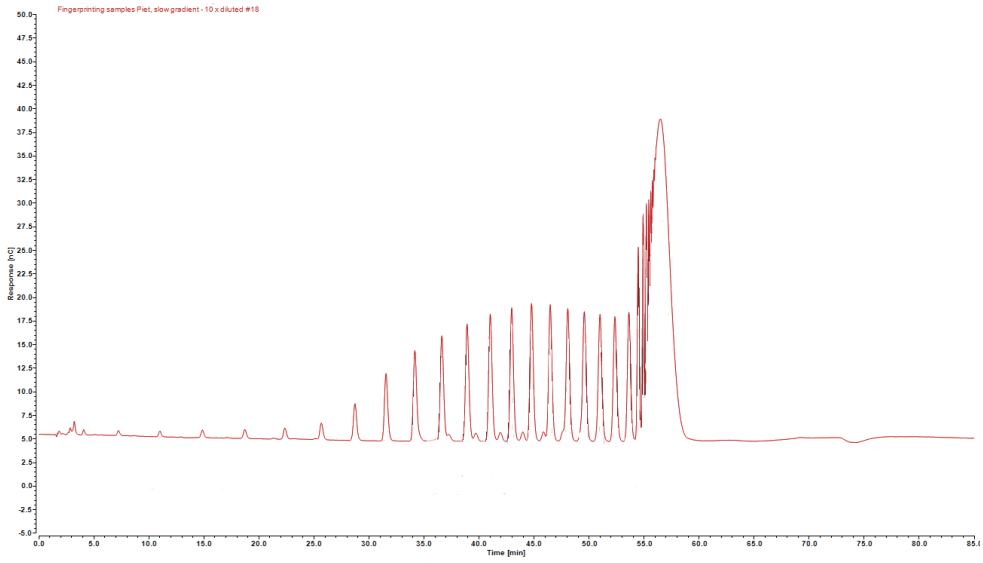

Figure S1. Long-chain inulin-type fructan (lcITF) HPAEC profile (red) compared to Frutafit DP10-60 reference (black). Peaks represent fructose (F) and glucose (G) monomers, dimers and fructans oligomers present in the formulation of lcITF. GFn and Fn chains respectively terminated by a glucose or fructose molecule with n the number of fructose moieties in the chain.

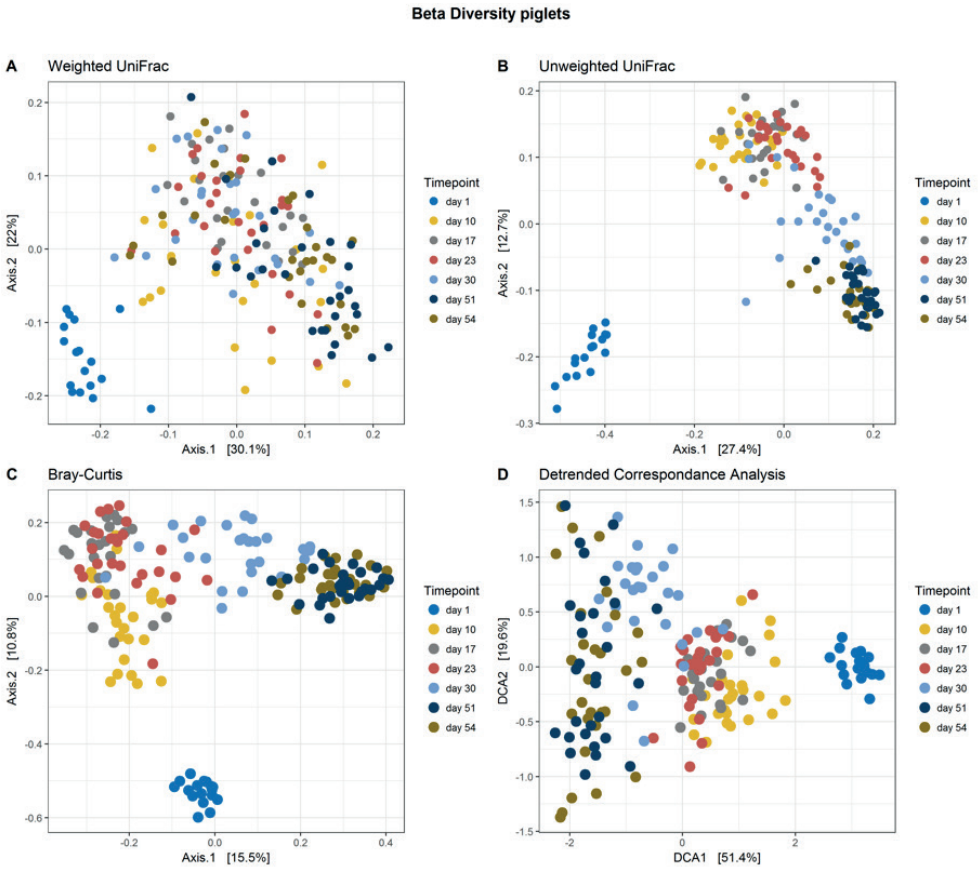

Figure S2. The microbiota development of neonatal piglets. Data were processed using A) the weighted and B) the unweighted Unifrac distances, C) Bray Curtis dissimilarity index and D) Detrended correspondance analysis using the Bray-Curtis distances.

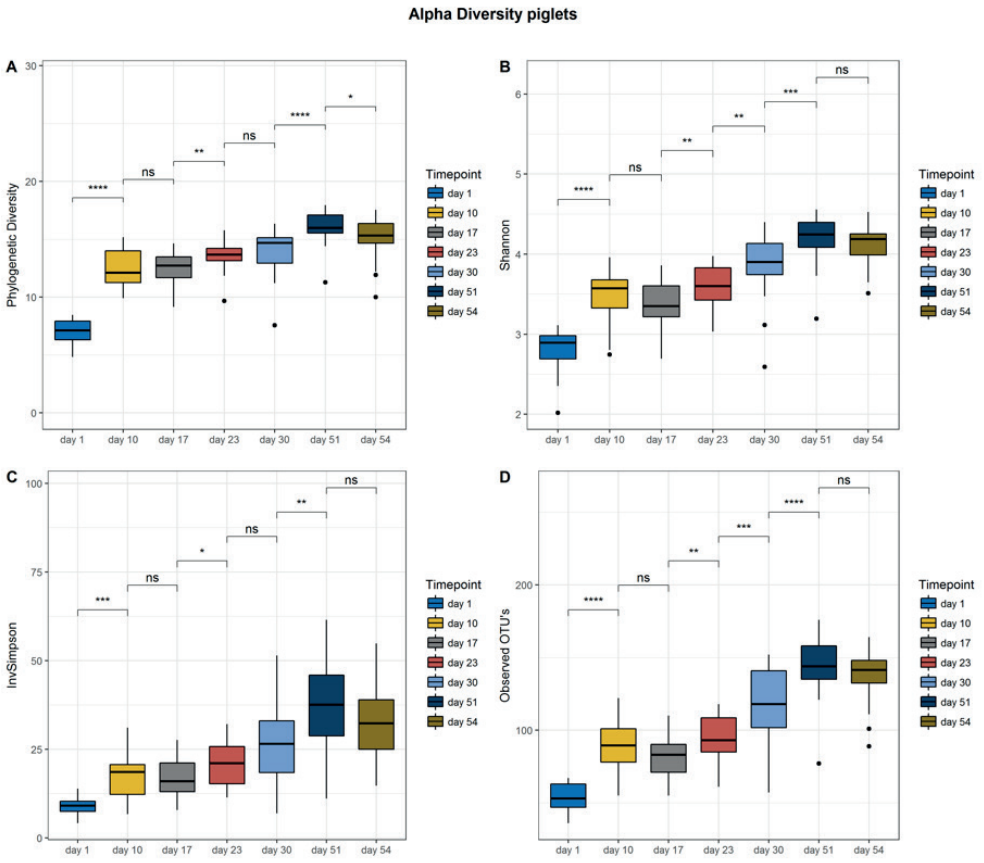

Figure S3. Alpha diversity calculations of neonatal piglets per timepoints. Alpha diversity was estimated using A) the Phylogenetic diversity index and B) the unweighted Unifrac distances, C) Bray Curtis dissimilarity index and D) Detrended correspondance analysis using the Bray-Curtis distances.

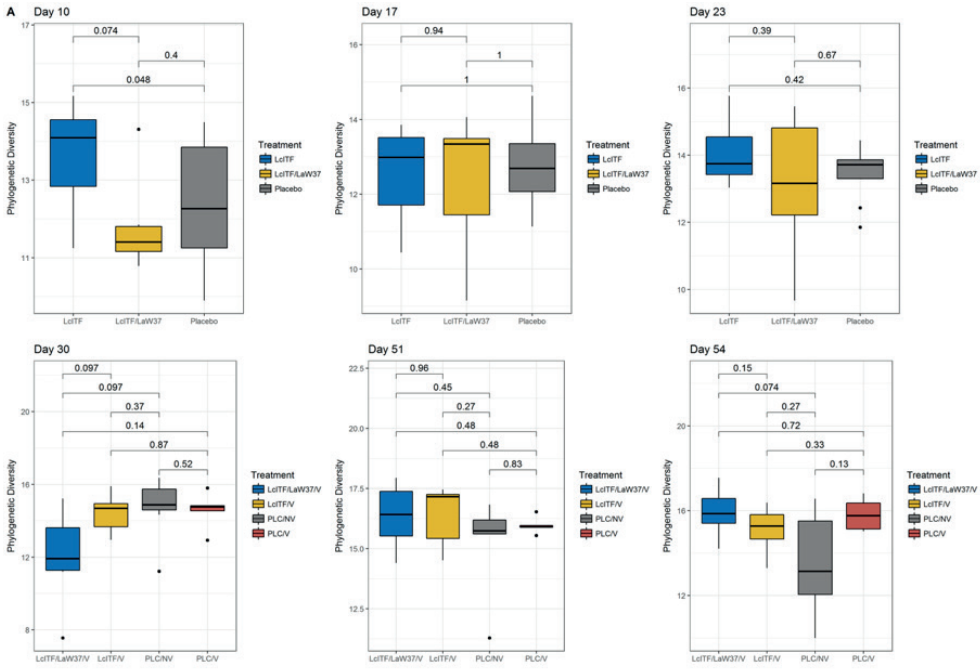

Figure S4. Alpha diversity measurements per group. Alpha diversity was estimated within each timepoint using the phylogenetic diversity index, for each of the groups, neamely CTRL/NV: control group receiving placebo and non-vaccinated; CTRL/V: control group receiving placebo and vaccinated; lcITF/V: intervention group receiving long-chain inulin type fructans and vaccinated; lcITF/LaW37/V: intervention group receiving the combination lcITF with *L. acidophilus* W37 and vaccinated.

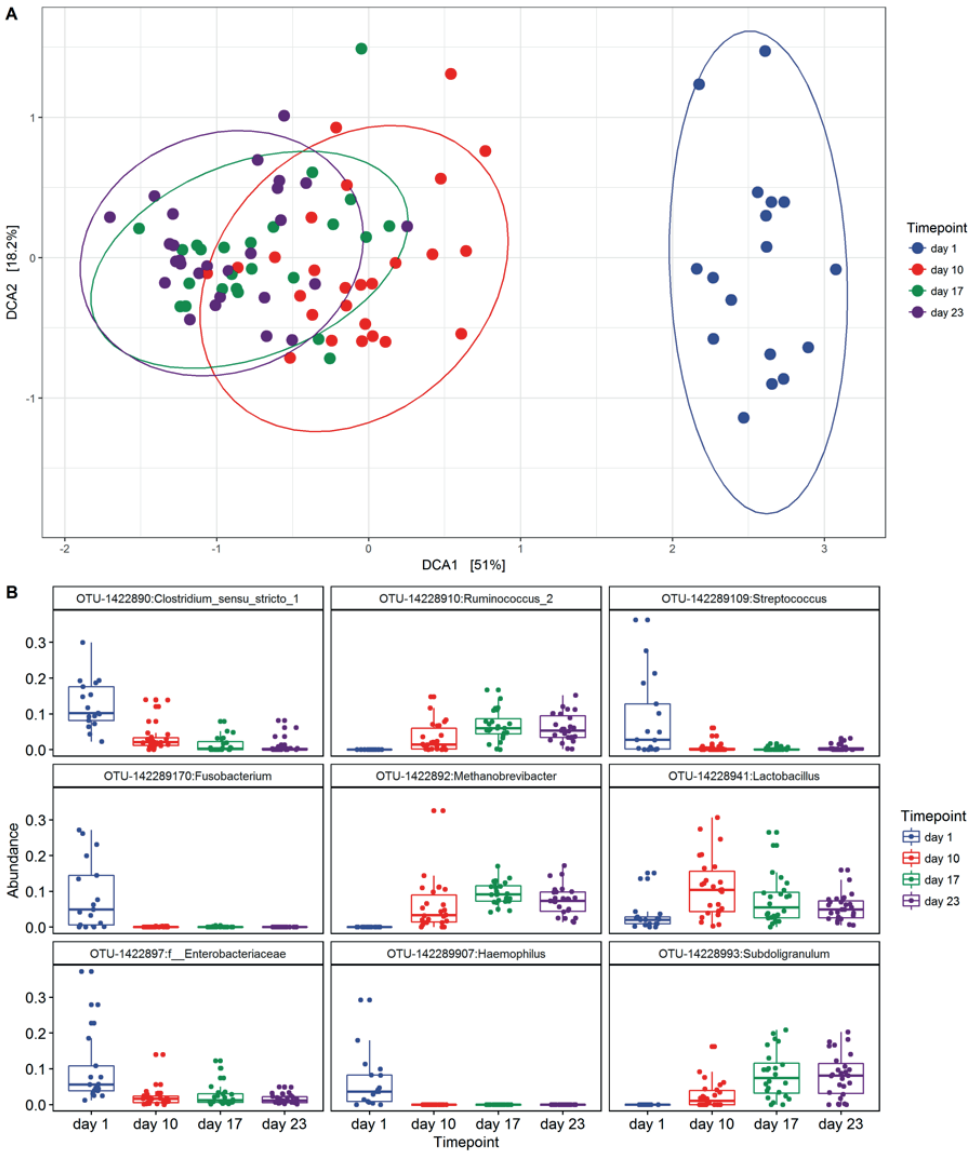

Figure S5. The development of piglet microbiota during preweaning. Graphs represent in A) DCA plot generated using the weighted Unifrac distances and in B) the OTU's with the highest coefficient of variation during preweaning.

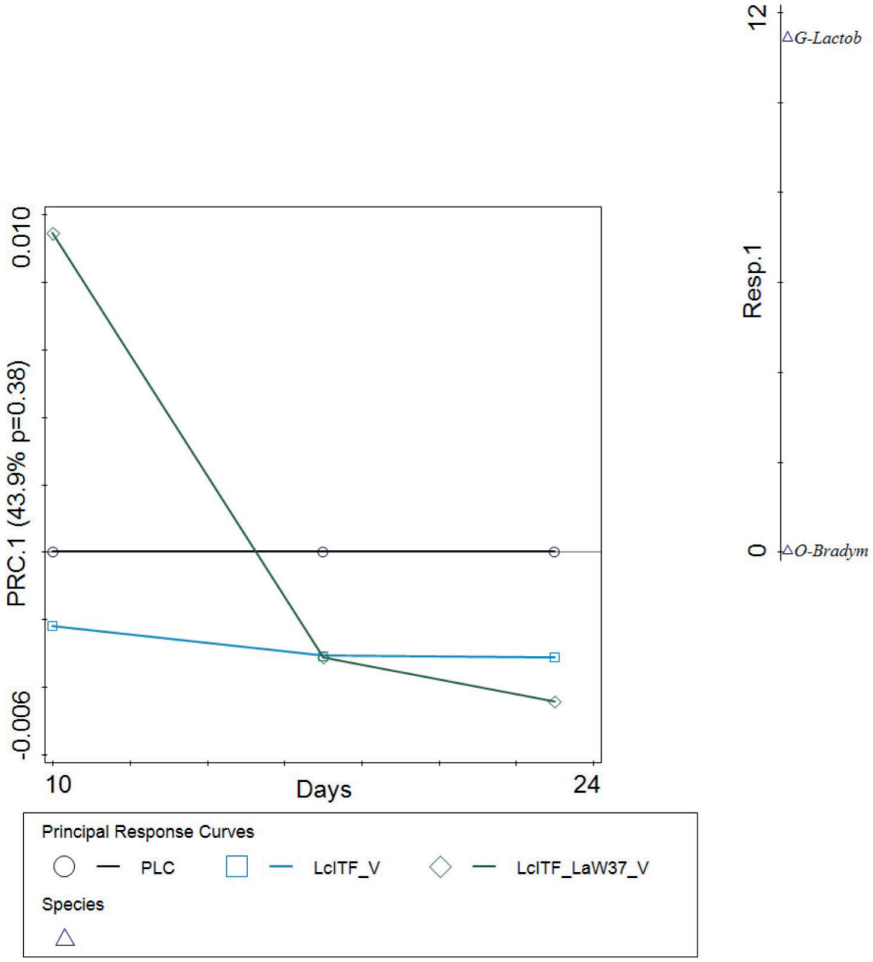

Figure S6. Principal response curve analysis prior to weaning. PRC testing for the effect of dietary intervention during the preweaning period revealed the microbial changes within samples collected on day 10, 17 and 21 after birth.

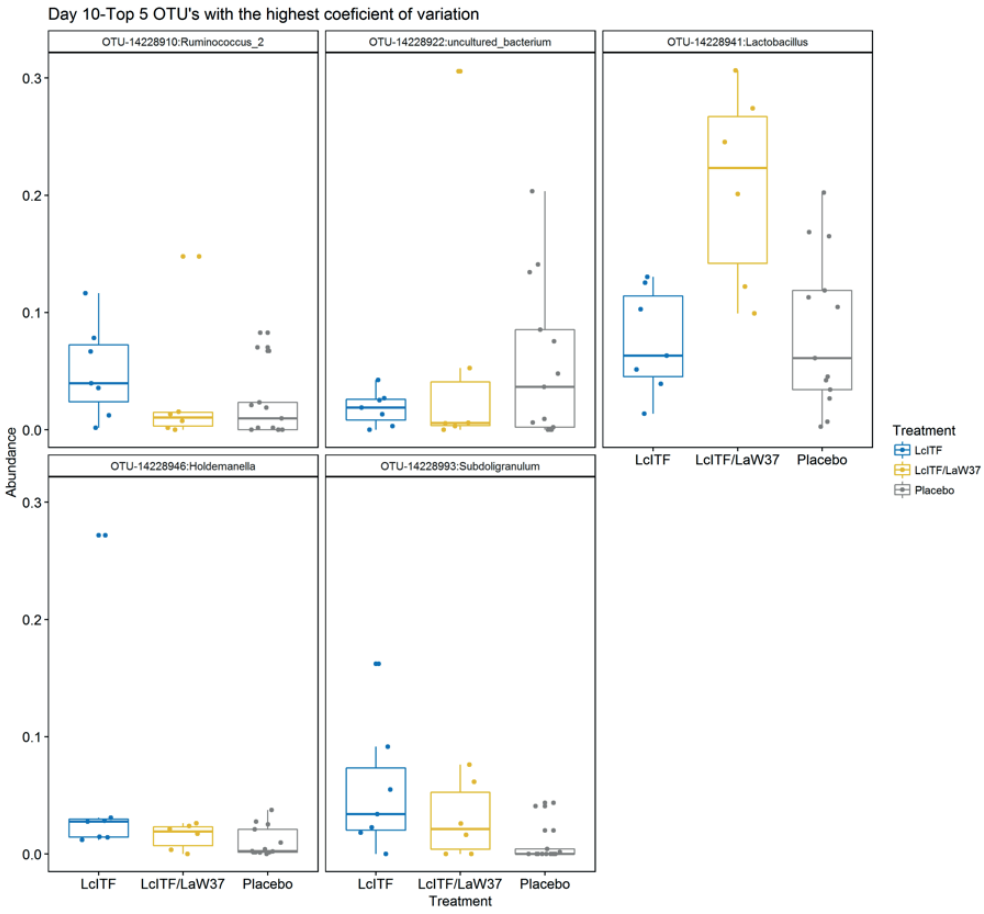

Figure S7. PREMANOVA analysis did not show significant effect for the variable treatment on day 10. Here we show the 5 taxa identified with the highest coefficients of variation.

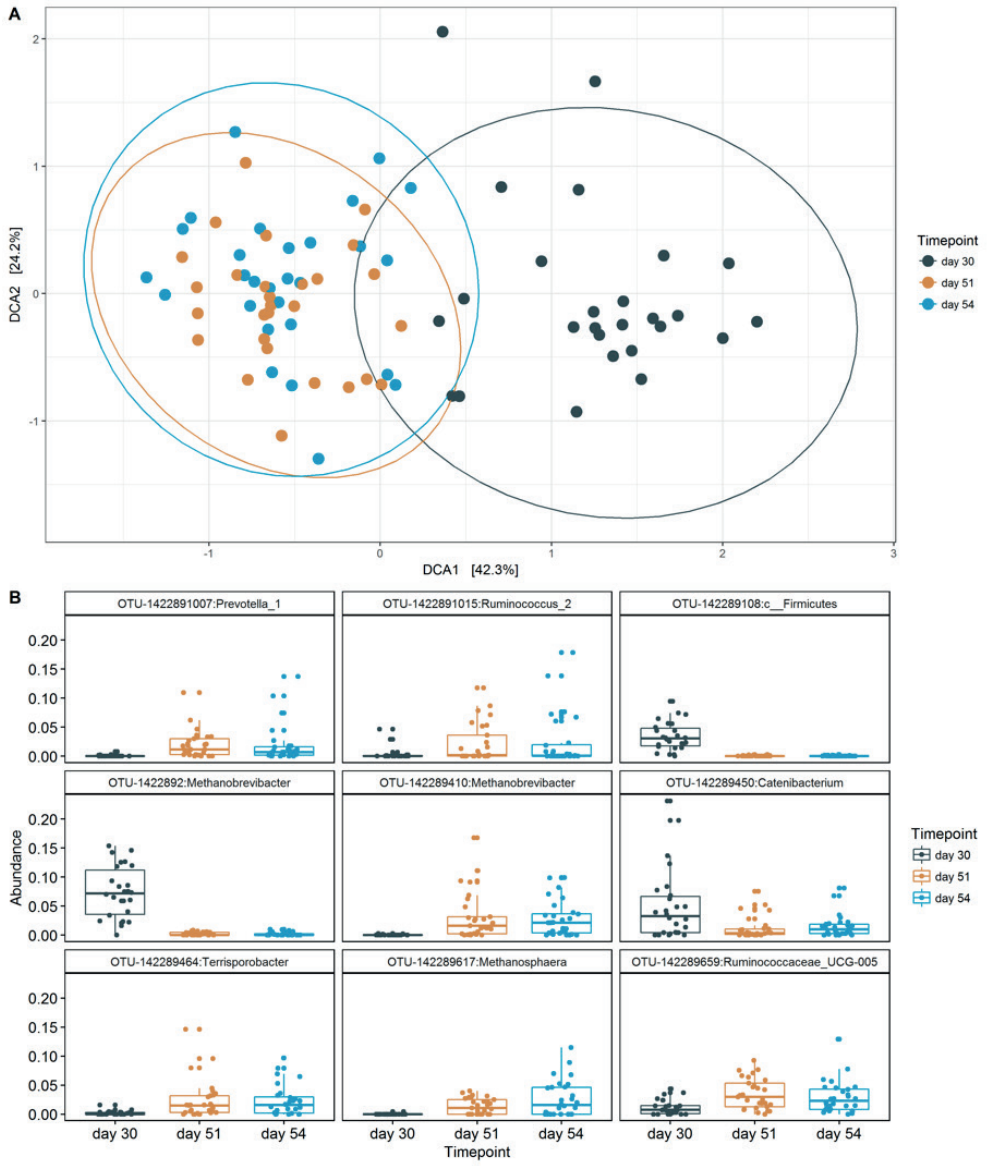

Figure S8. The development of piglets' microbiota post weaning. Graphs represent in A) the DCA plot generated using the weighted Unifrac distances and in B) the OTU's with the highest coefficient of variation during weaning on day 30, 51 and 54 after birth.

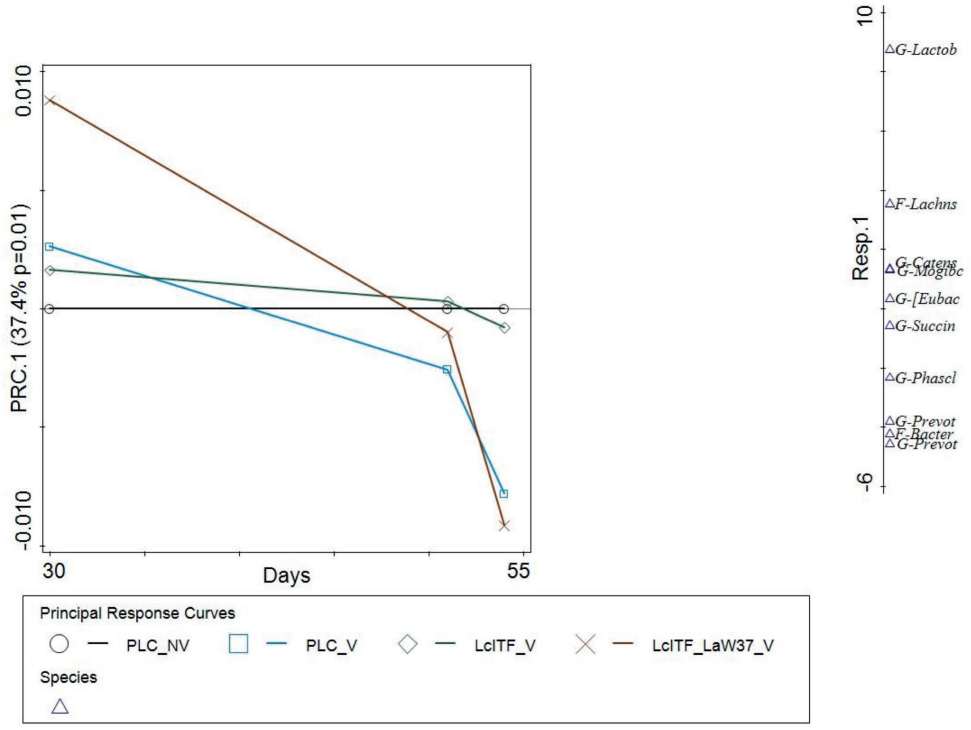

Figure S9. Principal response curve analysis post weaning. The PRC revealed microbial changes one week after weaning and upon infection using the samples collected on day 30, 51 and 54 after birth.

Tables

Table S1. Composition of the piglet weaner synthetic diet low in fiber.

Ingredient	%
Wheat starch native	50.2
Maize heat treated	10.0
Casein	8.8
Potato protein	4.2
Cellulose	5.0
Dextrose	2.0
Sugar	1.5
Soybean oil	4.2
Amino acids, minerals and vitamins	5.1

Table S2. Nutritional values of the synthetic diet for the swine net energy of 11.0 mega Joules/kg BW.

Nutrition component	%
Protein	18.9 %
Fat	5.1%
Fibre	4.3%
Ash	4.2%
Starch	50.9%
Non Starch Polysaccharides	8.0%
Sodium	0.3%
Calcium	0.6%
Phosphorus	0.5%
Lysine	1.3%
Methionine	0.6%

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